# STUDIES TOWARDS UNDERSTANDING THE FUNCTION OF LANCL1

BY

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THESIS

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## ABSTRACT

LanC-like protein 1 (LanCL1) is a eukaryotic homolog of bacterial protein LanC. Unlike the well studied LanC proteins, the functions of LanC-like proteins are yet unknown. To investigate the function of LanCL1 several studies were undertaken. Proteins interacting LanCL1 were tried to be found but were not accomplished. Also, the role of LanCL1 in apoptosis and oxidative stress were hypothesized, but the studies indicated no correlation. Finally, LanCL1 knockout mice were generated for further investigation.

## ACKNOWLEDGEMENTS

First, I would like to thank Dr. Wilfred van der Donk for giving me the opportunity to work in this laboratory and also always helping me and being a true advisor. I would have not been able to graduate without your help. Additionally, I would like to thank Dr. Jie Chen, also for giving me the opportunity to work in her laboratory and for her time. Also, I would like to thank the van der Donk group, Chen lab personnel and special individuals in my department. I had a wonderful time as a graduate student thanks to you. Finally, I would like to thank my family: my parents for supporting my pathway and for all the love that I received, and my brother for morale support through the tough times I had.

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# CHAPTER 1

# INTRODUCTION

Lantibiotics are lanthionine-containing antibiotic peptides, which are ribosomally synthesized and posttranslationally modified to their biologically active forms [1]. The posttranslational modifications involve the dehydration of Ser and Thr to dehydroalanine (Dha) and dehydrobutyrine (Dhb), which is followed by addition of Cys to the Dha and Dhb residues [1, 2]. The dehydration and cyclization reactions are catalyzed by LanB and LanC enzymes for class I lantibiotics. Eukaryotes contain Lanthionine synthetase component C-like proteins which are widely distributed among animals, plants, and insects. Eukaryotic LanC-like proteins have sequence identity with bacterial LanC proteins. However, in contrast to the known functions of LanC proteins in bacteria, very little is known about the function of LanC-like proteins in eukaryotes.

Humans have 3 homologs of LanC protein, LanC-like proteins 1, 2, and 3. Among these 3 proteins, LanC-like protein 1 (LanCL1) and 2 (LanCL2) are studied more compared to LanC-like protein 3 (LanCL3). LanCL1 is located on chromosome 2 [3], and was previously known as p40. Because of its seven hydrophobic domains, it

was initially characterized as a GPCR [4]. However, later it was found to be a peripheral membrane protein instead of an integral membrane protein and by refined sequence analysis, it was found that p40 contains a GxxG motif similar to LanC and was renamed to LanCL1 [5]. LanCL1 is highly expressed in the testis and brain [6], and it has been identified as a binding partner of *Plasmodium falciparum* PfSBP1 during malarial infection [7]. Also, it was reported as a novel binding partner for the redox regulatory compound glutathione, but no specific enzyme activities were observed *in vitro*, including GST activity, peroxidase activity, and catalyzing the conversion of GSH to GSSG or vice versa [8]. Most recently, the crystal structure of LanCL1 was reported, and it exhibits a double seven-helix barrel fold plus multiple GxxG motifs, which constitute the structural basis for Zn-dependent binding of GSH, similar to the structure of NisC [9]. In contrast, its homolog, LanCL2, is located on chromosome 7 [10], and is more ubiquitously expressed with an N-terminal myristoylation domain that associates with phosphoinositol phosphates [11]. LanCL3 is located on the X chromosome and no other studies have been reported so far [12].

Multiple roles of LanCL1 have been proposed, however, very little is known about the function of LanCL1 at the molecular and cellular levels. In this study, I have

explored the biological function of LanCL1 in HEK293 cells, in an effort to understand the significance of this protein.

# CHAPTER 2

# INVESTIGATION OF PROTEIN-PROTEIN INTERACTIONS OF LANCL1

## 2. 1 Introduction

One of the most powerful methods to gain information on a protein of unknown function is to look at its protein-protein interactions using a variety of biochemical, genetic and molecular biological techniques. Knowing its binding partners can help predict a protein's function [13]. LanCL1 has been reported as a binding partner of *Plasmodium falciparum* PfSBP1 during malarial infection. Also it was shown to bind to the SH3 domain of a signaling protein, Eps8 [9]. However, since LanCL1 shares high sequence homology with bacterial LanC (30%) [3], and the crystal structures are also similar, we raised the hypothesis that LanCL1 might catalyze similar reactions as LanC. To find out if there were any other binding partners for LanCL1, immunoprecipitation was used to identify novel interacting proteins.

## 2.2 Results

### 2.2.a LanCL1 does not interact with Akt or HSP90.

It was shown by Min Zeng in our laboratory that LanCL2 interacts with Akt and HSP90. Since LanCL1 is a homolog of LanCL2, the same co-immunoprecipitation experiment was performed for LanCL1. Flag-tagged LanCL1 was over expressed in HEK293 cells and immunoprecipitated with Flag antibody and the level of Akt and HSP90 was measured. However, no interactions were observed between LanCL1 and Akt and LanCL1 and HSP90 (Fig. 2.1).

### 2.2.b Flag immunoprecipitation of LanCL1 does not give any candidates for interacting proteins.

Previously in our laboratory, Min Zeng found that LanCL2 interacts with HSP90 by immunoprecipitation, using stably expressed LanCL2, silver staining, and mass spectrometry. To find interacting proteins of LanCL1, the same experiment was performed. N-Flag-LanCL1 stably expressed in HEK293 cells were immunoprecipitated with beads that were conjugated with Flag antibody and the eluted proteins were run on SDS gel and silver stained. However, no protein interaction with LanCL1 was observed

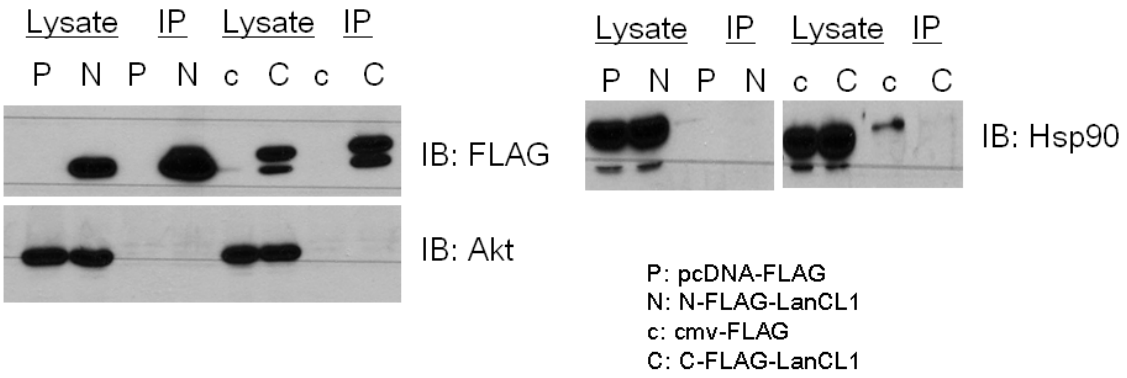



**Fig. 2.1** Co-immunoprecipitation of LanCL1 and Akt, HSP90. Akt and HSP90 do not interact with LanCL1. N and C terminally Flag tagged LanCL1 were transiently over expressed by transfection of HEK293 cells, then immunoprecipitated by Flag antibody, and the level of Akt and HSP90 was measured via Western blot. Lysates were loaded to confirm each protein was present before immunoprecipitation. pcDNA-Flag and cmv-Flag were used as negative controls for the N-Flag-LanCL1 and C-Flag-LanCL1.

(Fig. 2.2). The concentration of the detergent (Triton-X-100) could have been too high thereby disrupting protein-protein interactions so immunoprecipitation and silver staining was performed using different concentrations of Triton-X-100. However proteins interacting with LanCL1 could not be found (data not shown). The nature of the detergent (Triton-X-100 is a non-ionic detergent) could be one of the reasons why specific interactions with LanCL1 were not observed. Therefore, immunoprecipitation and silver staining were repeated with detergents that have different properties such as

CHAPS (zwitterionic detergent). However, interacting proteins of LanCL1 could still not be determined (data not shown).

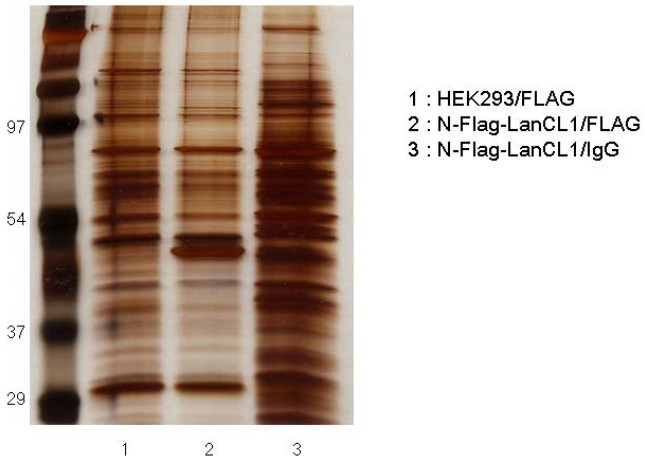



**Fig. 2.2** Silver staining after LanCL1 immunoprecipitation. No proteins that specifically interact with LanCL1 were found. Cells stably expressing N-Flag-LanCL1 were lysed and pre-cleared, and immunoprecipitated with Flag. Proteins were eluted and analyzed by SDS gel electrophoresis and silver stained. HEK293 cells were treated with Flag antibody and also N-Flag-LanCL1 stably expressing cells were treated with IgG as a negative control.

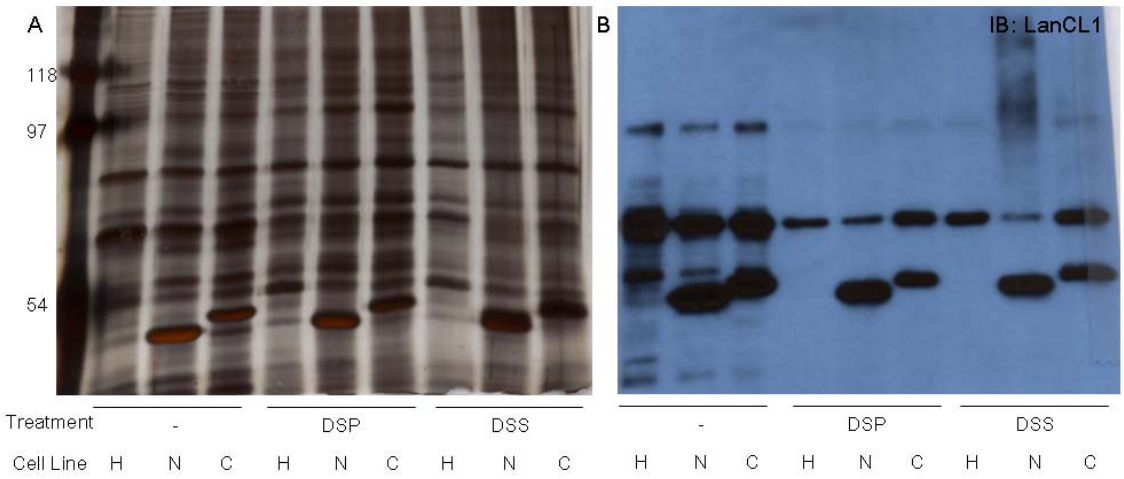
### 2.2.c Flag IP of LanCL1 in the presence of chemical crosslinkers does not give any candidates for interacting proteins.

The interaction with LanCL1 and other proteins might be relatively weak and easily broken by detergents. To prevent this, chemical crosslinkers, DSP (dithiobis[succinimidyl propionate]) and DSS (disuccinimidyl suberate) were used to covalently link proteins involved in protein-protein interactions. N-Flag-LanCL1 and C-Flag-LanCL1 stably expressed HEK293 cell lines were treated with DSP or DSS in PBS for 30 minutes prior to lysis, and after lysis immunoprecipitation and silver staining was performed. However, no protein interactions with LanCL1 were detected (Fig. 2.3). Also, LanCL1 stably expressed cells were lysed with DSP or DSS in the lysis buffer and immunoprecipitation and silver staining was performed. Once more no specific protein-protein interactions were observed with LanCL1 (data not shown).

### 2.2.d Establishment of C-Flag-LanCL1 stable cell lines.

HEK293 cells were transfected with C-terminal Flag tagged LanCL1 plasmid and the cells were selected in G418 containing media for several weeks. The expression level of LanCL1 was measured for 9 clones with LanCL1 antibody and most of them had high expression level. Three clones were picked; one that has higher expression level than endogenous LanCL1, one that has similar expression level with endogenous

LanCL1, and one that has lower expression level than endogenous LanCL1. These clones were expanded and frozen (Fig. 2.4).




H: HEK293 cells

N: N-Flag-LanCL1 stable cells

C: C-Flag-LanCL1 stable cells

**Fig. 2.3** Silver staining after LanCL1 immunoprecipitation using DSP and DSS. No proteins that specifically interact with LanCL1 were found using these chemical crosslinkers. (A) LanCL1 stable cells were treated with DSP or DSS in PBS for 30 minutes and lysed, immunoprecipitated, and silver stained. (B) Proteins that were eluted after immunoprecipitation were analyzed by SDS gel electrophoresis and blotted with LanCL1 antibody to see any shift. No shift was found.

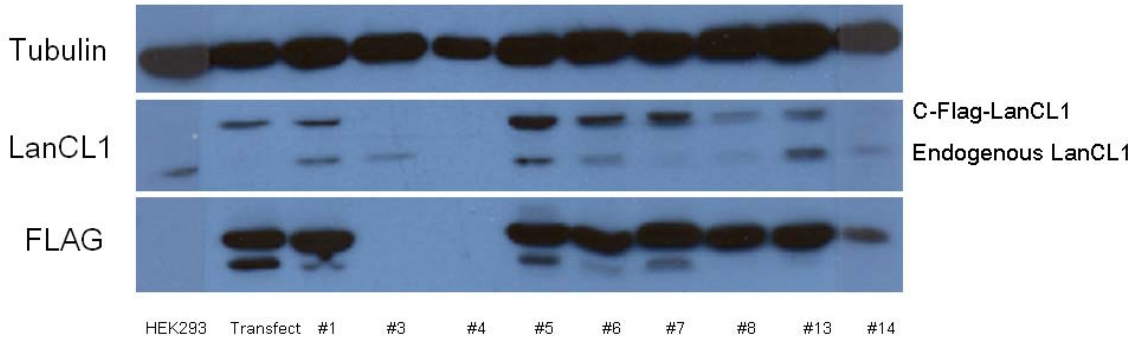



**Fig. 2.4** Western blot of C-Flag-LanCL1 stable cell lines. C-terminal Flag tagged LanCL1 were generated and tested for LanCL1 expression level. Cell line #5 was selected for higher expression level of C-Flag-LanCL1 compared to endogenous LanCL1, line #13 for similar expression level, and line #14 for higher endogenous expression level of LanCL1 than C-Flag-LanCL1.

## 2.3 Discussion

No proteins were found that were interacting with LanCL1 using immunoprecipitation, even with the use of chemical crosslinkers. However, this does not mean that LanCL1 does not have a binding partner, because many protein-protein interactions can not be detected by this technique.

# CHAPTER 3

# EFFECTS OF LANCL1 KNOCKDOWN OR OVEREXPRESSION

## 3.1 Introduction

Many signaling pathways are present in the cell, and many proteins are involved in these pathways [14-18]. It was shown in our laboratory that knocking down LanCL2 increased the level of phospho-Akt, and phospho-Erk. Since LanCL1 is a homolog of LanCL2, its function might be similar.

Akt is involved in various functions, such as cell survival, cell cycle, metabolism, and angiogenesis [19-22]. Among these functions, Akt is mostly known to function as a cell survival protein. It has been reported that phospho-Akt blocks apoptosis [23]. Phosphorylated Akt phosphorylates BAD, resulting in dissociation of BAD from the Bcl-2/Bcl-X complex. This process results in loss of pro-apoptotic function, leading to cell survival [24]. As will be shown in this chapter LanCL1 knockdown increases the level of phospho-Akt. Also it was found by our lab that LanCL2 knockdown cells are more resistant to induced apoptosis. Again with the same

assumption that LanCL1 might function similar to LanCL2, LanCL1 might also be involved in the apoptosis pathway.

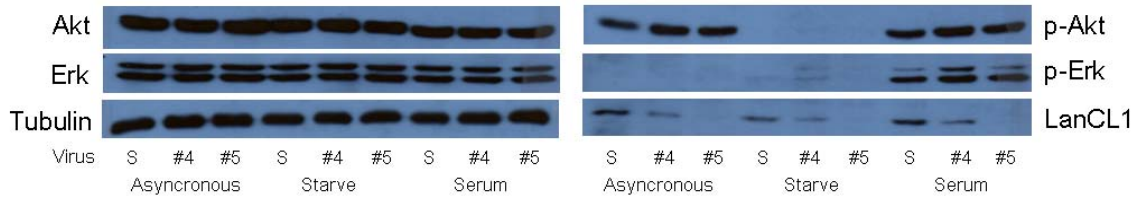
The fact that LanCL1 binds to glutathione might hint at a potential function of LanCL1. The major function of glutathione is as an antioxidant, neutralizing free radicals and reactive oxygen compounds. Although no specific enzymatic activities of LanCL1 using glutathione were reported [8], this observation could be the result of the experiments being performed *in vitro,* without many other cofactors that might be involved in the reaction that are present in the cell. Also, phospho-Akt levels increase when treated with $H_2O_2$ in HEK293 cells [25]. All together, LanCL1 could function in the regulation of oxidative stress.

Whole genomic expression patterns might also give us hints of LanCL1's function. Using microarray technology, gene expression level comparison with cells after scramble infection and LanCL1 knockdown will provide us genes that are upregulated or downregulated. Comparing the functions of these proteins with LanCL1 might lead to the function of LanCL1.

## 3.2 Results

### 3.2.a LanCL1 knockdown increases the level of phosphorylation in Akt (S473) and Erk.

It was shown by Min Zeng in our laboratory that LanCL2 knockdown induces increased level of phosphorylated Akt (S473) and Erk. To investigate if the same outcome is observed with LanCL1 knockdown, the same knockdown experiment was performed. LanCL1 was knocked-down and treated in three different conditions; asynchronous, starved, and serum treated. After the treatment, cells were lysed and blotted and analyzed with antibodies against different phosphorylated proteins. Phospho-Akt (S473) levels increased in asynchronous and serum treated condition and phospho-Erk levels increased only under serum treated conditions (Fig. 3.1). However, other phospho-proteins, including phospho-STAT3 (S727), phospho-S6K1, phospho-PKC, phospho-p38 and phospho-Jnk levels did not change (data not shown).




S: Scramble virus

#4, #5: LanCL1 knockdown virus

**Fig. 3.1** Western blot of phospho-Akt and phospho-Erk in asynchronous, starved and serum treated conditions after LanCL1 knockdown. LanCL1 knockdown increases phospho-Akt and phospho-Erk levels. LanCL1 was knocked-down by 2 independent viruses and the cells were subjected to asynchronous, starved and serum treated conditions. Phospho-Akt and phospho-Erk levels were measured by Western blot. Phospho-Akt levels increase in asynchronous and serum treated condition and phospho-Erk levels increase only in serum treated condition. Total Akt, total Erk and tubulin were measured as internal standards and LanCL1 was measured to determine the knockdown efficiency.

### 3.2.b LanCL1 and LanCL2 knockdown effect on the level of phosphorylation of Akt (S473) is not additive, nor synergistic.

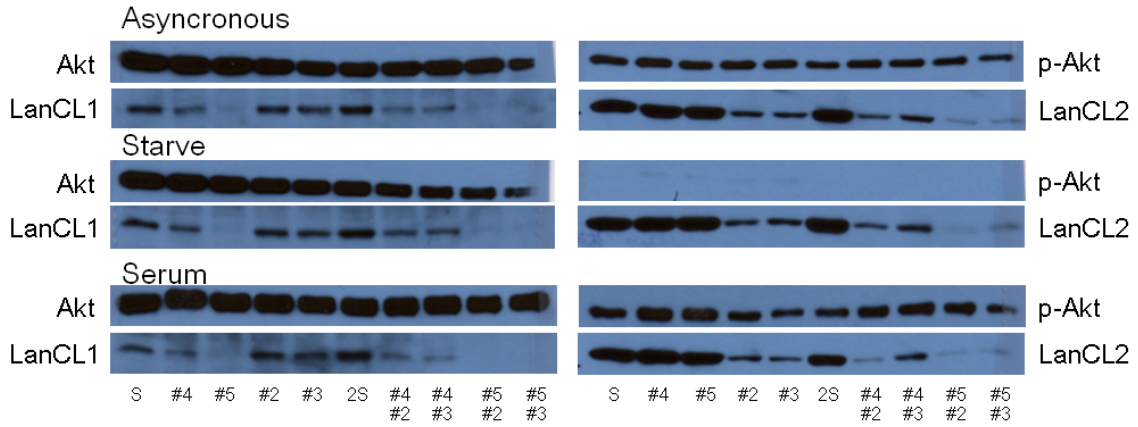
LanCL1 and LanCL2 knockdown has the same effect in increasing phospho-

Akt level. This observation could be the result of LanCL1 and LanCL2 being in the same pathway and compensating each other. To verify this hypothesis, LanCL1 and LanCL2 were knocked-down together and the cells were treated in three different conditions; asynchronous, starved, and serum treated. After the treatment, cells were lysed and phospho-Akt levels were measured. Neither an additive, nor synergistic effect was observed (Fig. 3.2), which means that LanCL1 and LanCL2 work in separate pathways.

### 3.2.c LanCL1 knockdown does not increase or decrease the level of DNA fragmentation when apoptosis is induced.

The major role of Akt is in cell survival. The increase of phospho-Akt with LanCL1 knockdown suggests that LanCL1 inhibits phospho-Akt formation, which could lead to cell death, apoptosis. Also, it was shown by Min Zeng in our laboratory that LanCL2 knockdown cells are resistant to induced apoptosis. DNA fragmentation levels were measured using TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay after LanCL2 knockdown and the level of DNA fragmentation decreased. To verify if LanCL1 is also involved in apoptosis, LanCL1 was knocked-

down, and apoptosis was induced by starving the cells for 2 days and treating with TNF-α and cycloheximide for 24 hours, and the TUNEL assay was performed. No change was observed in the DNA fragmentation level (Fig. 3.3).




S: Scramble virus
2S: Double amount of scramble virus
#4, #5: LanCL1 knockdown virus
#2, #3: LanCL2 knockdown virus

**Fig. 3.2** Western blot of phospho-Akt with asynchronous, starved and serum treated conditions after LanCL1 and LanCL2 double knockdown. Phospho-Akt levels do not respond in an additive or synergistic manner when LanCL1 and LanCL2 are knocked-down together. Phospho-Akt levels were measured by western blot after LanCL1, LanCL2, and LanCL1 with LanCL2 were knocked-down in three different conditions; asynchronous, starve, and serum treated. Total Akt was measured as a negative control and LanCL1 and LanCL2 levels were measured for the knockdown efficiency.

### 3.2.d LanCL1 knockdown or overexpression does not increase or decrease the level of cleaved caspase-3 when apoptosis is induced.

Min Zeng had shown that knockdown of LanCL2 decreases the level of cleaved caspase-3 levels after apoptosis is induced. LanCL2 was knocked-down and apoptosis was induced by using etoposide treatment for 2 days or starvation for 2 days, followed by treatment with TNF-α and cycloheximide for 24 hours, and the level of caspase-3 was measured. To see if LanCL1 has the same property, the same experiment was performed. LanCL1 was knocked-down and apoptosis was induced by the use of etoposide, or starvation and TNF-α, cycloheximide treatment. However, no difference was observed in the cleaved caspase-3 level (Fig. 3.4). Also, by using cells that stably express LanCL1, the same experiment was performed to see if there was any difference in the cleaved caspase-3 level when LanCL1 is over expressed. Again, no difference was observed (Fig. 3.5). However, when LanCL2 is over expressed in etoposide treatment, cleaved caspase-3 levels decreased, which was opposite to the predicted result.

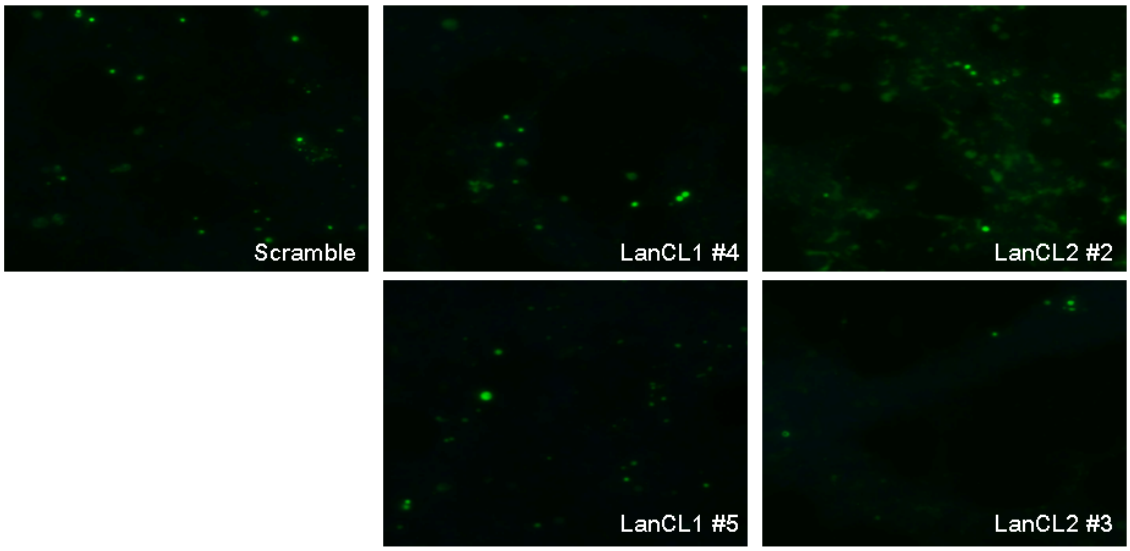



**Fig. 3.3** TUNEL assay of LanCL1 and LanCL2 knockdown after induced apoptosis. No difference in the DNA fragmentation level was observed when LanCL1 was knocked-down compared to scramble. LanCL1 and LanCL2 were knocked-down and apoptosis was induced by starving cells for 2 days and treating with TNF-α and cycloheximide for 24 hours and TUNEL assay was performed. Two independent viruses were used for both LanCL1 and LanCL2 to rule out off target effects. Scramble was used as a negative control and LanCL2 knockdown was used as a positive control.

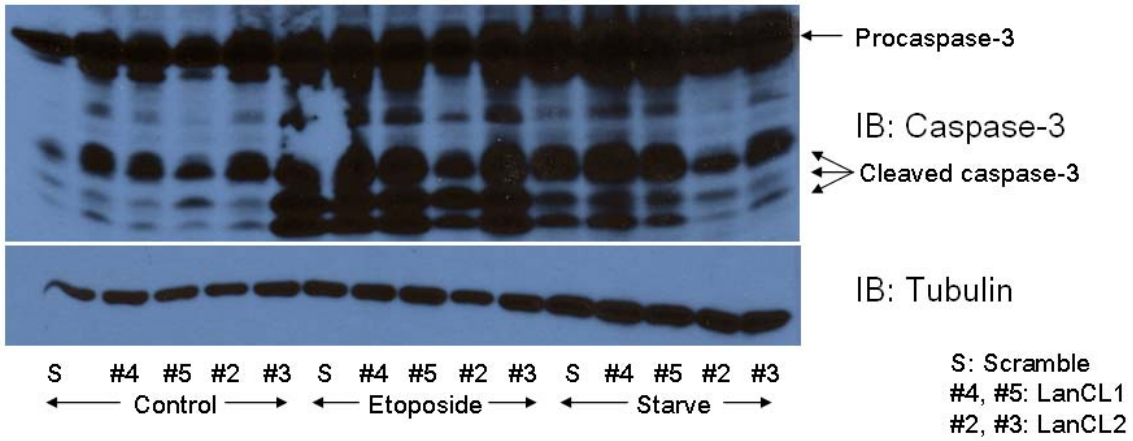



**Fig. 3.4** Western blot of caspase-3 upon induced apoptosis after LanCL1 or LanCL2 knockdown. Cleaved caspase-3 levels do not change when LanCL1 is knocked-down and apoptosis is induced. LanCL1 was knocked-down using 2 independent viruses and apoptosis was induced by etoposide treatment for 2 days or starvation for 2 days and treatment with TNF-α and cycloheximide for 24 hours. Caspase-3 level was measured by Western blot. Tubulin and a Lentivirus containing a scramble sequence was used as a negative control and viruses targeting LanCL2 were used as a positive control. However, LanCL2 knockdown with etoposide treatment did not show the same effect with starved condition. IB = Immunoblot

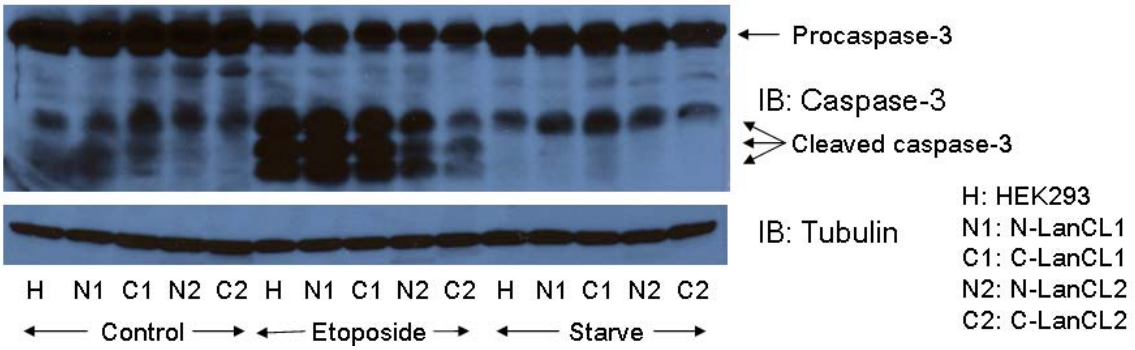



**Fig. 3.5** Western blot of caspase-3 after inducing apoptosis in cells over expressing LanCL1 or LanCL2. Cleaved caspase-3 levels do not change when LanCL1 is over expressed and apoptosis is induced. LanCL1 was over expressed using N-Flag-LanCL1 and C-Flag-LanCL1 stable cell lines and apoptosis was induced by etoposide treatment for 2 days or starvation for 2 days and treatment with TNF-α and cycloheximide for 24 hours. Caspase-3 level was measured by Western blot. Tubulin and HEK293 cells were used as internal standard and a negative control, respectively. Starvation condition seemed not to induce apoptosis in this experiment but in other experiments (2 out of 3), results for cleaved caspase-3 level were the same with etoposide treatment.

### 3.2.e LanCL1 knockdown or overexpression does not increase or decrease the level of apoptosis rate when apoptosis is induced.

If LanCL1 is involved in apoptosis, the rate of apoptosis would be different

when LanCL1 is knocked-down or over expressed. To test this hypothesis, the rate of apoptosis was measured by knocking down or over expressing LanCL1, treating with etoposide to induce apoptosis, and measuring the cell viability for 6 consecutive days by MTT assay starting from day 0. No difference between the two experiments was observed (Fig. 3.6).

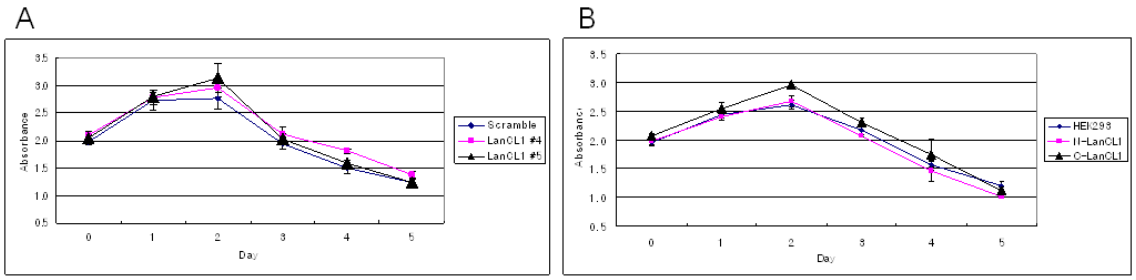



**Fig. 3.6** MTT assay of induced apoptosis after LanCL1 knockdown and over expression. The apoptosis rate was not different when LanCL1 was knocked-down or over expressed. (A) LanCL1 was knocked-down using 2 independent viruses and treated with etoposide for 5 days. MTT assay was performed each day to measure cell viability. (B) LanCL1 was over expressed using N-Flag-LanCL1 and C-Flag-LanCL1 stable cell lines. MTT assay was performed using the same method as described above. Scramble and HEK293 cells were used as a negative control.

### 3.2.f LanCL1 level does not change after starvation and serum stimulation.

If LanCL1 is involved in apoptosis, the level of LanCL1 may change upon induction of apoptosis. To see if LanCL1 levesl change during apoptosis process, cells were starved, and the level of LanCL1 was measured, and increase in LanCL1 levels were observed (Fig 3.7.A). To confirm that the LanCL1 decrease was due to the serum starvation, cells were treated with serum after starvation, and the level of LanCL1 was measured. However, after serum treatment, LanCL1 level did not decrease (Fig 3.7.B).

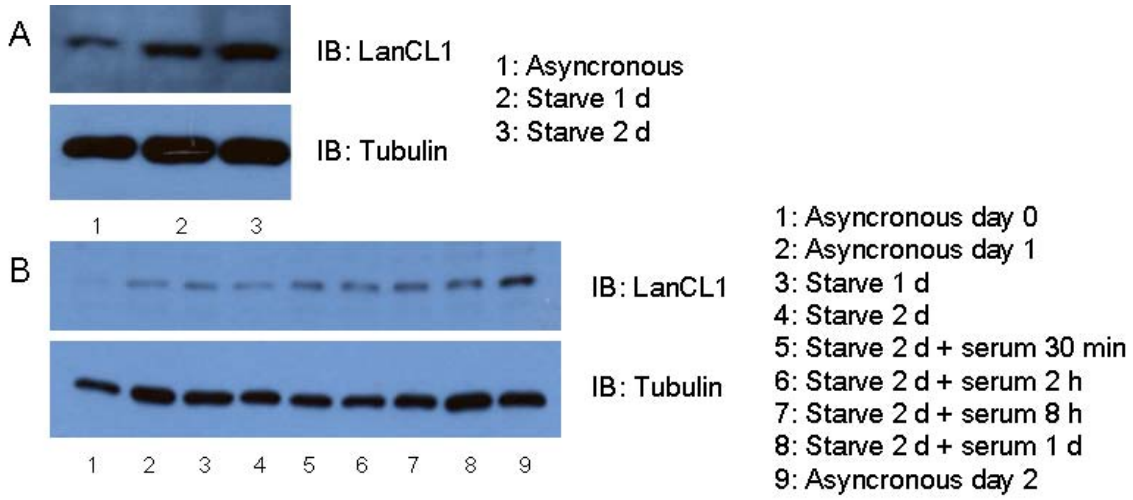



**Fig 3.7** Western blot of LanCL1 after serum starvation and stimulation in HEK293 cells. LanCL1 level increases upon starvation but after serum stimulation there are no changes. (A) HEK293 cells were starved for 0, 1 day or 2 days. LanCL1 level was measured by Western blot. (B) HEK293 cells were starved for 0, 1 day or 2 days. After 2 days of serum starvation, cells were treated with FBS for 30 min, 2 h, 8 h or 1day. LanCL1 level

was measured by Western blot. Tubulin was used as internal standard.

### 3.2.g LanCL1 knockdown or overexpression does not increase or decrease the cell viability with $H_2O_2$ induced oxidative stress.

Glutathione is an antioxidant that neutralizes free radicals and reactive oxygen compounds. Since LanCL1 binds to glutathione, LanCL1 might be involved in the oxidative stress response *in vivo*. To verify this hypothesis, cell viability was measured by MTT assay after LanCL1 was knocked-down or over expressed and treated with $H_2O_2$, a reactive oxygen compound, in different concentrations (Fig. 3.8). When LanCL1 was knocked-down, and N-Flag-LanCL1 was over expressed, there was no difference in cell viability compared to a control with scramble virus. However, when C-Flag-LanCL1 was over expressed, cell viability at 1 mM $H_2O_2$ increased, compared to wild-type HEK293 cells.

### 3.2.h The level of increase in phosphor-Akt (S473) after LanCL1 knockdown is not correlated with $H_2O_2$ induced oxidative stress.

LanCL1 knockdown increases phospho-Akt level. Also phospho-Akt level increases when HEK293 cells are subjected to $H_2O_2$. To verify any correlation with $H_2O_2$ treatment and LanCL1, LanCL1 was knocked-down and treated with different concentrations of $H_2O_2$ for 24 hours and phospho-Akt levels were measured. However, the results were not consistent and overall, no correlation was found (Fig. 3.9).

### 3.2.i Microarray and statistical analysis after LanCL1 knockdown does not correlate with gene functions.

Whole genome expression pattern might hint us in LanCL1's function. To find genes that are upregulated or downregulated when LanCL1 is absent, LanCL1 was knocked-down using two independent Lentiviruses in HEK293 cells. RNA was extracted and genome expression pattern was obtained using microarray. Statistical analysis showed that 19 genes were downregulated and 10 genes were upregulated (Table 2.1). Each downregulated or upregulated genes were clustered by function using DAVID (Database for Annotation, Visualization and Integrated Discovery). Downregulated genes showed correlation with apoptosis and upregulated genes showed no significant correlation (Table 2.2). These results contradict previous results

indicating that LanCL1 has no correlation with apoptosis. However, the genes that function in apoptosis showed that some upregulate apoptosis (DNA-damage-inducible transcript 3, phorbol-12-myristate-13-acetate-induced protein 1, tumor protein p53 inducible nuclear protein 1) and some downregulate apoptosis (hepatitis B virus x interacting protein).

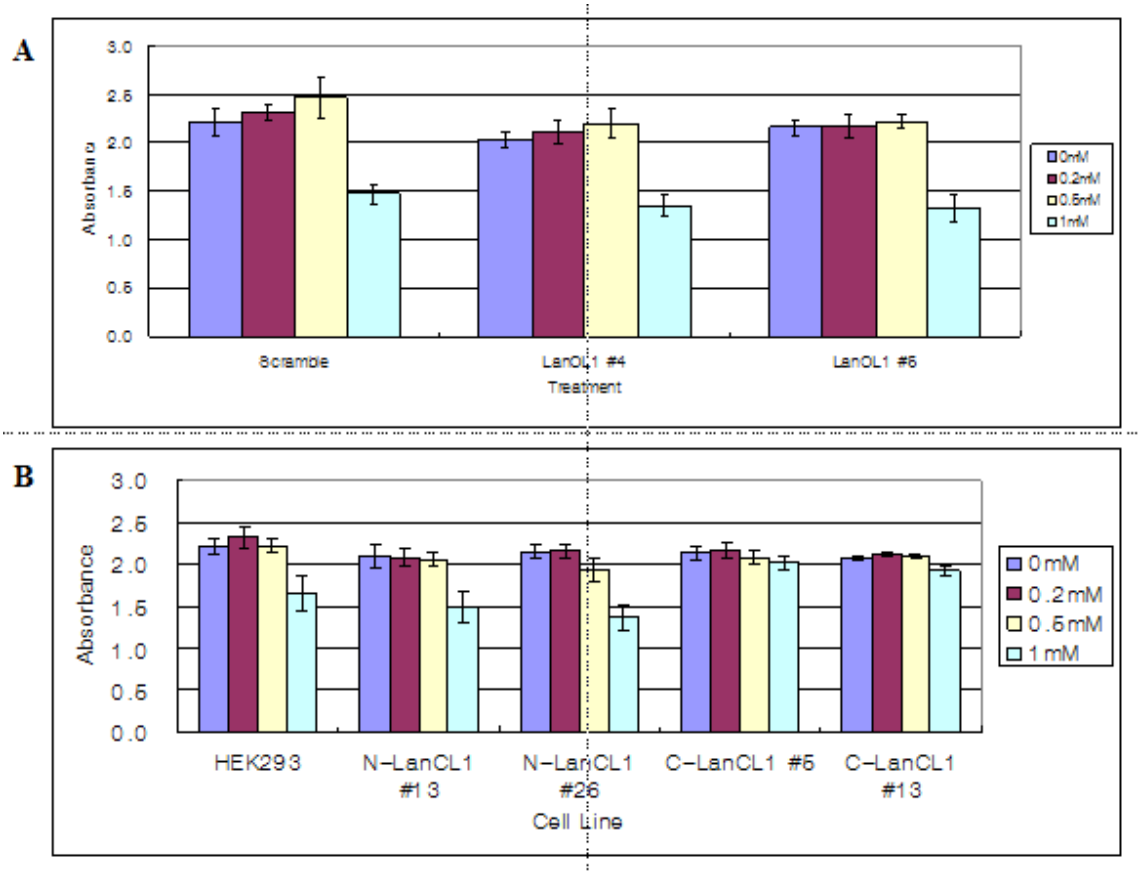



**Fig. 3.8** MTT assay of $H_2O_2$ treatment after LanCL1 knockdown or over expression. (A) LanCL1 was knocked-down using 2 independent viruses and treated with 0, 0.2, 0.5 and 1 mM $H_2O_2$ for 24 h, and cell viability was measured. (B) LanCL1 was over expressed

using N-Flag-LanCL1 and C-Flag-LanCL1 stably expressed cell lines and treated with 0, 0.2, 0.5 and 1 mM $H_2O_2$ for 24 h and cell viability was measured. A Lentivirus with a scramble sequence and HEK293 cell were used as negative controls.

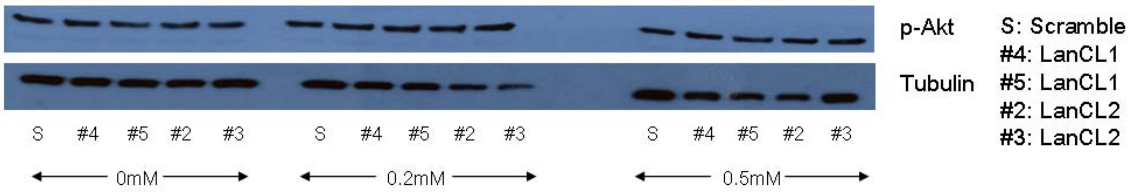



**Fig. 3.9** Western blot for phospho-Akt with $H_2O_2$ treatment after LanCL1 or LanCL2 knockdown. LanCL1, LanCL2 were knocked-down using 2 independent viruses each and the knockdown cells were treated with 0, 0.2, 0.5, 1 mM $H_2O_2$ for 24 h and phospho-Akt level was measured. Tubulin was used as a internal standard. 1 mM $H_2O_2$ treated samples are not shown because the cells died.

| Gene ID | Probe sets | Downregulated probe sets | Significance overall | Proportion of significance |
|---|---|---|---|---|
| LanC lantibiotic synthetase component C-like 1 | 12 | 12 | 12 | 1.00 |
| Ret finger protein-like 4A | 2 | 2 | 2 | 1.00 |
| DNA-damage-inducible transcript 3 | 5 | 3 | 4 | 0.80 |
| Leukocyte immunoglobulin-like receptor | 9 | 5 | 7 | 0.78 |
| Protein phosphatase 1, regulatory (inhibitor) subunit 15A | 4 | 1 | 3 | 0.75 |
| Deoxycytidine kinase | 10 | 6 | 7 | 0.70 |
| Zinc finger, AN1-type domain 2A | 5 | 1 | 3 | 0.60 |
| Nuclear RNA export factor 1 | 24 | 5 | 14 | 0.58 |
| tRNA-yW synthesizing protein 3 homolog | 7 | 5 | 4 | 0.57 |
| Phorbol-12-myristate-13-acetate-induced protein 1 | 7 | 3 | 4 | 0.57 |
| Tumor protein p53 inducible nuclear protein 1 | 7 | 2 | 4 | 0.57 |
| CDP-diacylglycerol synthase 1 | 16 | 10 | 8 | 0.50 |
| Hepatitis B virus x interacting protein | 6 | 4 | 3 | 0.50 |
| TAF3 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 140kDa | 8 | 2 | 4 | 0.50 |
| Glycolipid transfer protein | 6 | 2 | 3 | 0.50 |
| Histone cluster 1, H4l | 4 | 1 | 2 | 0.50 |
| Gap junction protein, delta 4, 40.1kDa | 2 | 1 | 1 | 0.50 |
| Smith-Magenis syndrome chromosome region, candidate 8 | 2 | 1 | 1 | 0.50 |
| Zinc finger protein 98 (F7175); | 2 | 1 | 1 | 0.50 |

| Gene ID | Probe sets | Upregulated probe sets | Significance overall | Proportion of significance |
|---|---|---|---|---|
| Calcitonin-related polypeptide beta | 5 | 2 | 5 | 1.00 |
| Nephroblastoma overexpressed gene | 7 | 5 | 6 | 0.86 |
| Neuritin 1 | 4 | 2 | 3 | 0.75 |
| Protein phosphatase 1, regulatory (inhibitor) subunit 15A | 4 | 1 | 3 | 0.75 |
| Delta/notch-like EGF repeat containing | 15 | 10 | 11 | 0.73 |
| Limbic system-associated membrane protein | 8 | 2 | 5 | 0.63 |
| Tenascin C | 28 | 13 | 17 | 0.61 |
| Guanidinoacetate N-methyltransferase | 9 | 4 | 5 | 0.56 |
| Matrix metallopeptidase 2 | 15 | 4 | 8 | 0.53 |
| Melanoma cell adhesion molecule | 17 | 5 | 9 | 0.53 |

**Table 3.1** Downregulated and upregulated genes after LanCL1 knockdown in HEK293 cells. LanCL1 was knocked-down using 2 independent viruses in HEK293 cells. RNA was extracted and using microarray, downregulated and upregulated genes (p-value<0.05) were identified. Each gene consisted of probe sets, and the number of downregulated or upregulated probe sets and significance overall was obtained. Number of significance overall was divided by number of probe sets for proportion of significance. Only genes that have a proportion of significance over 0.5 were counted and 19 genes were downregulated and 10 genes were upregulated.

A.

| Term | Count | % | P-Value |
|---|---|---|---|
| cell cycle arrest | 3 | 13.6 | 4.90E-03 |
| mutagenesis site | 7 | 31.8 | 6.50E-03 |
| nucleus | 9 | 40.9 | 2.10E-02 |
| cellular response to unfolded protein | 2 | 9.1 | 2.20E-02 |
| endoplasmic reticulum unfolded protein response | 2 | 9.1 | 2.20E-02 |
| response to endoplasmic reticulum stress | 2 | 9.1 | 3.50E-02 |
| ER-nuclear signaling pathway | 2 | 9.1 | 3.60E-02 |
| Apoptosis | 3 | 13.6 | 4.40E-02 |
| regulation of apoptosis | 4 | 18.2 | 4.70E-02 |
| regulation of programmed cell death | 4 | 18.2 | 4.80E-02 |
| regulation of cell death | 4 | 18.2 | 4.80E-02 |

B.

| Term | Count | % | P-Value |
|---|---|---|---|
| signal | 8 | 66.7 | 1.00E-04 |
| signal peptide | 8 | 66.7 | 1.10E-04 |
| disulfide bond | 7 | 58.3 | 5.80E-04 |
| disulfide bond | 7 | 58.3 | 6.80E-04 |
| glycoprotein | 7 | 58.3 | 5.70E-03 |
| domain:EGF-like 9 | 2 | 16.7 | 1.10E-02 |
| domain:EGF-like 7 | 2 | 16.7 | 1.30E-02 |
| cell adhesion | 3 | 25 | 1.60E-02 |
| skeletal muscle fiber development | 2 | 16.7 | 1.60E-02 |
| domain:EGF-like 5 | 2 | 16.7 | 1.90E-02 |
| domain:EGF-like 6 | 2 | 16.7 | 2.00E-02 |
| muscle fiber development | 2 | 16.7 | 2.10E-02 |
| extracellular matrix | 3 | 25 | 2.30E-02 |
| domain:EGF-like 4 | 2 | 16.7 | 2.70E-02 |
| glycosylation site:N-linked (GlcNAc...) | 6 | 50 | 2.70E-02 |
| striated muscle cell development | 2 | 16.7 | 3.00E-02 |
| muscle cell development | 2 | 16.7 | 3.30E-02 |
| domain:EGF-like 3 | 2 | 16.7 | 3.50E-02 |
| synapse organization | 2 | 16.7 | 3.60E-02 |
| Secreted | 4 | 33.3 | 3.80E-02 |
| skeletal muscle organ development | 2 | 16.7 | 3.80E-02 |
| skeletal muscle tissue development | 2 | 16.7 | 3.80E-02 |
| domain:EGF-like 2 | 2 | 16.7 | 4.10E-02 |

**Table 3.2** Expected function of downregulated and upregulated genes. Each

downregulated or upregulated gene was clustered into related functions using DAVID. (A) Expected function of downregulated genes. (B) Expected function of upregulated genes. Functions that have a p-value lower than 0.05 were selected. Count is the number of genes that are related to that function and % is count divided by total number of genes.

## 3.3 Discussion

LanCL1 knockdown also increases the level of phospho-Akt and phospho-Erk similar to LanCL2 knockdown. However, the increase is stronger with LanCL1 #4 virus even though the knockdown efficiency is lower compared to LanCL1 #5 virus. Also, according to the double knockdown, LanCL1 and LanCL2 seem to not compensate each other. Nevertheless, increase in the level of phospho-Akt could have been maximized by a single knockout, explaining why a double knockdown shows the same effect as single knockdown.

The observation that there was no difference in DNA fragmentation level, cleaved caspase-3 level and apoptosis rate when LanCL1 was knocked-down or over

expressed showed that LanCL1 is not involved in the apoptosis pathway, even though statistical analysis of microarray data suggested implication in apoptosis because the statistical analysis showed both upregulating and downregulating genes of apoptosis. Additionally, LanCL1 was not totally knocked out and the residual LanCL1 might still be capable of its function showing the weakness of the microarray experiment. Also, the result that there was no difference in cell viability when LanCL1 was knocked-down or over expressed compared to scramble or HEK293 cells, and the fact that there was no correlation with the increase of phospho-Akt level when LanCL1 was knocked-down and oxidative stress induced by $H_2O_2$ increase in phospho-Akt level, showed that LanCL1 was also not correlated with oxidative stress.

# CHAPTER 4

# GENERATION OF LANCL1 AND LANCL3 KNOCKOUT MICE

## 4.1 Introduction

Knockout mice are a good method in understanding the function of a protein in vivo. A protein's physiological function in vivo could be determined by targeted gene disruption using Zinc Finger Nucleases (ZFNs), which are a class of engineered DNA-binding proteins that facilitate targeted editing of the genome by creating double-strand breaks (DSBs) in DNA at specified locations [26]. After creation of DSBs, endogenous DNA repair processes repair DNA by non-homologous end joining [27], which leads to frameshifts and results in targeted gene knockout. Generation of LanC-like protein knockout mice would be a powerful model in understanding its function.

## 4.2 Results

### 4.2.a Generation of founder LanCL1 and LanCL3 knockout mice.

ZFN for LanCL1 was designed to bind the third exon and was generated to randomly cut among 5 nucleotides between ZFN binding sites (Fig 3.1). ZFN for LanCL3 was designed to bind the first exon and was generated to randomly cut among 6 nucleotides between ZFN binding sites (Fig 4.1). ZFN mRNA was microinjected in FVB mice embryos at pronuclear stage before transferring to pseudo pregnant females. Genotyping by PCR and surveyor assay confirmed 8 LanCL1 knockout litters among 73 (Fig 4.2), and 20 LanCL3 knockout litters among 114 (Fig 4.3).

LanCL1: CCCTCGGGATGGCACtgggtACACTGGCTGGGCAG

LanCL3: CGCTGCTTCGCCAACcgcttcGATGACTACCAGGGCAGCC

**Fig 4.1** LanCL1 and LanCL3 ZFN binding and cutting site. Red fonts indicates the intended cutting site.

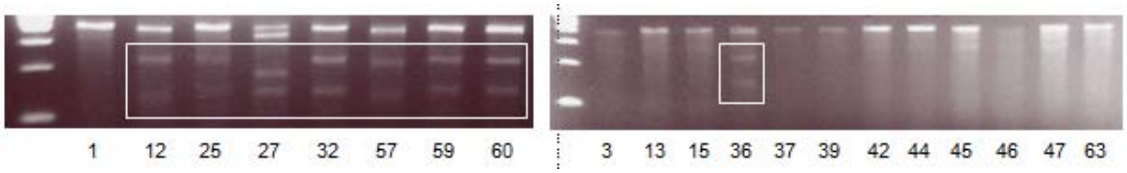



**Fig 4.2** Genotyping results of LanCL1 ZFN microinjected litters. Numbers indicate the numbers for each mice.

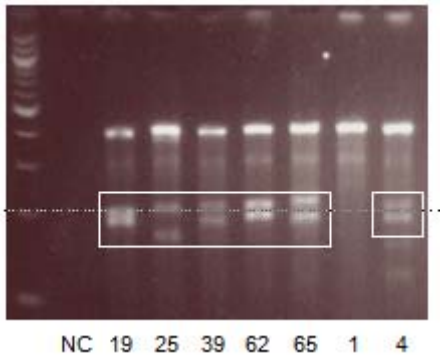



**Fig 4.3** Genotyping results of LanCL3 ZFN microinjected litters. Numbers indicate the numbers for each mice.

### 4.2.b Selection of LanCL1 and LanCL3 knockout mice line.

The obtained LanCL1 and LanCL3 knockout founder mice were not heterozygous and were mosaic. To generate LanCL1 and LanCL3 heterozygous mice, mosaic LanCL1 or LanCL3 knockout founder mice were bred with wild type mice. With genotyping by PCR and surveyor assay, LanCL1 and LanCL3 heterozygous mice were confirmed. ZFN target area DNA for each mouse line was amplified by PCR (LanCL1 forward primer: TCCATATGTGGTTTCTGAAAAGC, LanCL1 reverse primer: AGCGCCAGGCATGAATAC, LanCL3 forward primer: GTCTTGTCACCTCCCGTCTC, LanCL3 reverse primer: GCTCTGGGAGACGTGGTAGA) and were sequenced to determine the actual

nucleotide deletions (LanCL1 - #25: 1bp, #27: 48bp, #32: 5bp, #36: 19bp, #57: 15bp, #59: 2bp and #60: 6bp deletion/ LanCL3 - #15: 6bp, #19: 12bp, #32: 2bp, #67: 37bp, #89: 1bp, #104: 2bp deletion). Because of the need of frameshift mutants, line 36 for LanCL1 and line 67 for LanCL3 were selected for further generation of LanCL1 and LanCL3 knockout mice. LanCL1 and LanCL3 heterozygous mice were bred with each other to generate knockout mice.

### 4.2.c LanCL1 protein is not detected in LanCL1 knockout mice.

Two of each wild-type, LanCL1 heterozygous and LanCL1 knockout mice were sacrificed. Brain, heart, liver and skeletal muscle were harvested. Each organ was lysed and LanCL1 level was measured by western blot (Fig 4.4). LanCL1 protein was not detected in LanCL1 knockout mice.

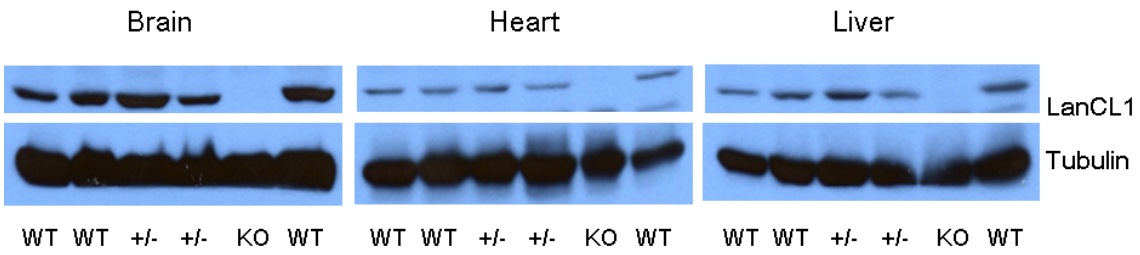



**Fig 4.4** Western blot of LanCL1 in brain, heart and liver of wild-type, LanCL1 heterozygous and LanCL1 knockout mice.

## 4.3 Discussion

Generation of LanCL1 knockout mice will allow the application of many methods to understand its function. General phenotype, overall survival, fertility, and development can be examined. Typical organs of LanCL1 knockout mice can be compared by histology. Also, behavioral experiments such as measuring motor or memory activities could be performed. With knockout LanCL1 mice established in this work, such studies can now be undertaken.

# CHAPTER 5

# MATERIALS AND METHODS

## Cell culture:

HEK293 cells were maintained in growth media (DMEM containing 10% FBS (Thermo), 1% L-glutamine (Cellgro), 1% sodium pyruvate (Cellgro), 1% penicillin/streptomycin(Cellgro)) at 37 °C in 5.5% $CO_2$. Starvation media contained no FBS in media and serum activation contained 30% FBS.

## Transfection of HEK293 cells:

Prior to transfection, media was changed to fresh 0.5 mL growth media. DMEM (50µl-12 well, 100µl-6well), DNA (3µg LanCL1), and polyfect (QIAGEN) were mixed and incubated at RT for 5-10 minutes, and then added to cells drop by drop.

## Knockdown of LanCL1, LanCL2:

Prior to infection, media was changed to 0.5 mL fresh growth media with polybrene (Sigma, 0.008 mg/ml). After that, scramble, LanCL1 #4, LanCL1 #5, LanCL2 #2 and LanCL2 #3 virus was added to the media. After cells were confluent, they were split

in growth media with puromycin (Sigma, 0.15μl/mL).

**Immunoprecipitation:**

Cells were lysed in MIPT lysis buffer (50 mM Tris-Cl (Fisher) pH 7.2, 100 mM NaCl (Fisher), 25 mM NaF (Sigma), 25 mM β-glycerophosphate (Sigma), 2 mM EDTA (Fisher), 2 mM EGTA (Fisher), 10 mM sodium pyrophosphate (Fisher)) with 0.3% Triton-X-100 (Fisher), and 1:100 protein inhibitor cocktail (Sigma). Lysate was centrifuged at 13000 rpm at 4 °C for 10 minutes. Supernatent was incubated with Flag antibody conjugated beads (Sigma) for 30 minutes. The beads were washed 3 times with lysis buffer and boiled in 2XSDS sample buffer. When immunoprecipitated proteins were subjected to silver staining, 0.1, 0.3, 0.5, 0.7, and 1.0% Triton-X-100 or 0.1, 0.3, 0.6, and 1.0% CHAPS (Sigma) were used in lysis buffer and after 15000 x g centrifugation, the supernatant was centrifuged at 150000 x g for 1 h, pre-cleared using IgG conjugated beads (Sigma) for 1 h twice, and incubated with Flag antibody conjugated beads for 30 min. The beads were washed with lysis buffer 3 times and supernatant comtaining 3XFlag peptides (Sigma or from our protein science facility) were incubated with the beads for 30 min, 2 times, all at 4°C. The eluted proteins was boiled in 2XSDS sample buffer.

**Immunoprecipitation using chemical crosslinkers:**

Prior to cell lysis, cells were washed with PBS once and treated with 1 mg/ml DSP (Thermo, 10% DMSO (Fisher)) or 1 mg/ml DSS (Thermo, 10% DMSO) in PBS for 30 min. After treatment, the cells were washed again with PBS once and were lysed with RIPA buffer (40 mM HEPES (Fisher), 2 mM EDTA, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1% NP40 (Fisher), 0.1% SDS (Sigma)). The lysate was immunoprecipitated using the method above. Another method used was lysing cells using RIPA buffer with 1mg/ml DSP (10% DMSO) or 1 mg/ml DSS (10% DMSO) and performing immunoprecipitation using the method above.

**Western blot:**

Protein samples that are in 2XSDS sample buffer are loaded on 7.5%, 10%, or 12% SDS gel with loading dye. Once the loading dye runs out, protein electrophoresis was stopped and the gel was transferred to a PVDF (Millipore) membrane for 70 min at 250 mA. After the transfer, the membrane was blocked with 5% skim milk (Nestle) in PBST (0.05% Tween-20 (Fisher) in phosphate buffered saline) for 30 min and incubated with primary antibody (HSP90 (Cell signaling) 1:500, Akt (Cell signaling) 1:1000, Erk (Cell

signaling) 1:1000, Flag (Sigma) 1:10000, Tubulin (Abcam) 1:10000, phospho-Akt (S473) (Cell signaling) 1:1000, phospho-Erk (Cell signaling) 1:1000, phospho-Jnk (Cell signaling) 1:1000, phospho-STAT3 (S727) (Cell signaling) 1:500, phospho-S6K1 (Cell signaling) 1:1000, phospho-PKC (Cell signaling) 1:1000, phospho-p38 (Cell signaling) 1:1000, LanCL1 (Abnova) 1:1000, LanCL2 (our lab) 1:1000, caspase-3 (Cell signaling) 1:1000 over night at 4 °C. Next day, the membrane was washed with PBST 3 times for 10 min each and incubated with secondary antibody (Goat anti-mouse or anti-rabbit (Jackson Immunoresearch)) for 30 min at RT. The membrane was washed 3 times, 10 min each with PBST and developed using Plus-ECL enhanced chemiluminescence substrate (Perkin Elmer) and exposed to films.

**Silver staining:**

After SDS PAGE gel electrophoresis, the gel was fixed in fixing buffer (50% methanol (Fisher) and 12% acetic acid (Fisher)) overnight. Next day, the gel was washed 3 times in water for 10 min each, then sensitized in STS buffer (0.02% (m/v) $Na_2S_2O_3$ (Fisher)) for 1 min. The gel was washed 2 times with water 1 min each and soaked in staining solution (44 mL $H_2O$, 5.9 mL $AgNo_3$ (Fisher, 0.1M) and 37.5µl formaldehyde (Fisher, 37% (w/w)) for 30 minutes. After that, the gel was washed in water for less than 1

minute and put in developing solution (100ml 3% $Na_2CO_3$ (Fisher), 2ml STS buffer, and 50ul formaldehyde (37% (w/w))) until protein bands were clearly seen. Once protein bands were seen, the reaction was stopped by 12% acetic acid.

**Establishment of C-Flag-LanCL1 stable cell lines:**

HEK293 cells were transfected with cmv (cytomegalovirus) -Flag-LanCL1 construct and were selected in G418 (Sigma) containing media for 24 h. The transfected cells were split (40-50 cells per 10 cm plate) and grown until they formed colonies that were visible by naked eye. By using a sterile filterpaper soaked in trypsin (Cellgro), all colonies were transferred to a 48-well plate and grown in G418 containing media. After cells were confluent, the level of LanCL1 was measured by western blot.

**Inducing apoptosis:**

Apoptosis was induced by two methods. 1. The media was changed to serum free media for 2 days and treated with TNF-α (1:1000) and cycloheximide (1:1000) for 24 h. 2. The cells were directly treated with etoposide (Sigma, 1:1000) for 2 days.

**TUNEL assay:**

TUNEL assay was performed by the protocol by Promega. After apoptosis was induced, cells were fixed with 4% formaldehyde in PBS for 25 min. After that, the cells were washed with PBS for 5 min, 2 times. The cells were permeabilized in 0.2% Triton-X-100 in PBS for 5 min and washed again with PBS for 5 min, 2 times. Using TUNEL assay kit (Promega), equilibration buffer was added for 10 min and after that, rTdT incubation buffer was added for 1 h at 37 °C. The reaction was stopped by adding 2XSSC buffer for 15 min and cells were washed with PBS 5 times, and incubated with DAPI (1:1000) in 3% BSA in PBS. The cells were washed again with PBS 3 times and then mounted on slides.

**MTT assay:**

MTT assay was performed by the protocol by Invitrogen. Cells were split in 96-well plate. On the day of experiment, MTT assay buffer (Invitrogen) was added (1:10) to the media for 5 hours at 37°C with 5.5% $CO_2$. After that, the media was aspirated and 200 ul DMSO was added to dissolve the formazan and were incubated for 5 min at 37 °C with 5.5% $CO_2$. The absorbance was read at 520 nm with 96-well plate reader (Tecan).

**$H_2O_2$ treatment:**

On the day of treatment, media was changed to fresh growth media with $H_2O2_2$ (Fisher, 0.2, 0.5, 1.0 mM) for 24 h. After treatment, media was aspirated and the cells were lysed.

**Microarray analysis:**

LanCL1 was knocked-down in HEK293 cells using the method above. RNA was extracted by the protocol by Qiagen and with the microarray chip (Affimetrix, ST 1.0), was sent to Roy J. Carver Biotechnology Center (Univ. of Illinois, Urbana-Champain) for experiment and statistical analysis. Genes with p-value lower than 0.05 were selected. Among these genes, genes that have proportion of significance (number of significance/ number of probe sets) over 0.5 were selected.

**Extraction of DNA from mouse tails:**

Mouse tails were cut at postnatal 20 days and lysed in 0.6 mL genomic DNA isolation buffer (20 mM Tris-Cl pH 7.5, 50 mM EDTA, 100 mM NaCl, 0.5% SDS) and 15 μl proteinase K (NEB) for 8 h. 0.24 mL tail salt (4.21 M NaCl, 0.63 M KCl, 10 mM Tris-Cl pH 8.0) was added for 30 min at 4 °C. After 10 min 15000 x g centrifugation, supernatant was transferred and filled with 100% ethanol (Fisher) and centrifuged and 15000 x g for another 10 min. 1 mL 80% ethanol was added and centrifuged at 15000 x

g for 5 min. Pellet was dissolved in 0.2 mL TE (1 mM Tris-Cl pH 8.0, 0.1 mM EDTA)

**Generation of LanCL1 and LanCL3 knockout mice:**

LanCL1 and LanCL3 ZFN (Sigma) mRNA was microinjected in FVB mice (Jackson) embryos at pronuclear stage before transferring to pseudo pregnant females at Transgenic Mouse Facilty (TMF, Univ, of Illinois, Urbana-Champaign).

**Surveyor assay**

0.2 μL primer (Sigma), 0.5 μL dNTP (NEB), 2.5 μL Taq buffer (NEB), 0.2 μL Taq (NEB), dd$H_2O$ (20.4 μL for LanCL1 and 17.9 μL for LanCL3), DMSO (0 for LanCL1 and 2.5 μL for LanCL3) and 1 μL DNA were mixed and PCR (annealing temperature: 55 °C, 30 cycle) was performed. DNA hybridization is performed by 95 °C 2 min, 95 °C ramping to 85 °C (-2 °C/s), 85 °C ramping to 25 °C (-0.1 °C/s). Samples are treated with 1 μL surveyor enhancer S (Transgenomic) and 1 μL surveyor nuclease S (Transgenomic) for 1 h at 42 °C and run on 2% agarose (Lonza) gel.

# CHAPTER 6

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